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STUDIES ON THE ANAEROBIC BACTERIAL FLORA OF SUPPURATIVE PERIODONTITIS

A DISSERTATION SUBMITTED TO THE FACULTY OF THE DIVISION OF THE BIOLOGICAL SCIENCES IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BACTERIOLOGY AND PARASITOLOGY

1941

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ELIZABETH S. HEMMENS

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STUDIES ON THE ANAEROBIC BACTERIAL FLORA OF SUPPURATIVE PERIODONTITIS

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Although the characteristic pathological changes of suppurative periodontitis may be found in ancient skeletal remains, the earliest known record which can be associated with the disease according to Goadby[1] is comparatively recent. In 1550 Ambroise Paré, chief surgeon to the king of France, recommended surgical treatment of the condition characterized by loosening of the teeth. More easily recognizable descriptions were made by Fuchard in 1746 and Gendarme in 1778, when they noted suppuration of the alveoli and gums as an accompaniment to the bone resorption described by Paré. The term "alveolar pyorrhea," meaning a flow of pus from the alveolus, was first used by Toirac in 1779. Nearly 100 years later, in 1867, Riggs made what is considered to be a classical description of the disease and for a time his name was given to the condition which he described as necrosis of the alveolar process. Today the terms "pyorrhea alveolaris" and "suppurative periodontitis" cover several different diseases of the periodontal membrane which have been classified by Becks,[2] Box,[3] Kronfeld[4] and others according to differences in clinical, pathological and etiological characteristics. However, it is only recently that these attempts to clarify the clinical concept of these diseases have been made and their effect has only begun to be noticeable in the literature. The tendency in earlier studies was to regard these diseases as a single clinical entity with one specific cause. In the present investigation, the term "suppurative periodontitis" has been used to describe the cases from which material was taken for study, classification being made on the basis of the clinical findings.

The literature covering the various aspects of suppurative periodontitis is voluminous and, particularly in regard to the relation of bacteria and other microorganisms to the condition, is often contradictory. Probably one of the earliest and most extensive investigations is that of Goadby,[1] who studied smears and aerobic cultures from 100 persons having this disease, but he was not able to associate any one bacterial type with the process. On the basis of opsonic tests he concluded that periodontitis resulted from a lowered resistance due to insufficient production of immune substances against the normal flora of the mouth and that it was not a specific bacterial infection. Eyre and Payne,[5] Merritt,[6] Hitchens[7] and Smirnow[8] also favored this viewpoint. A further refinement which, however, was not supported by any clinical or experimental data, was added by Bertrand and Valodier[9] when they postulated a nutritional basis or deficient antibody

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1. Lancet. **1:** 633, 1907.
2. J. Am. Dent. A. **16:** 2167, 1929.
3. Canadian Research Foundation Bulletin no. 7, 1924; cited by Kronfeld.
4. Histopathology of the Teeth and Their Surrounding Structures, ed. 2, Philadelphia, Lea and Febiger, 1939, p. 316.
5. Proc. Roy. Soc. Med., Odont. Sect. **3:** 29, 1909–1910.
6. Dental Cosmos **51:** 44, 1910.
7. Dental Cosmos **57:** 1, 1915.
8. Dental Cosmos **57:** 1010, 1915.
9. Compt. rend. Soc. de biol. **75:** 432, 1915.

production. On the basis of Goadby's theory, these investigators and many others employed autogenous vaccines or vaccines made from various mouth organisms as a therapeutic measure in cases of suppurative periodontitis, but results were disappointing in the majority of instances and the method is no longer used. The basic hypothesis has never been definitely disproved but apparently few workers regard it seriously today.

From time to time there have been reports indicating that suppurative periodontitis is an infectious disease (Leary,[10] Needles[11]) with a specific microbic etiology. Among the causative organisms suggested have been staphylococci alone (Harris[12]), or combined with streptococci (Hartzell,[13] Fisher[14]), or with fusospirochetes (Grossett[15]). Smith and Barrett[16] considered that bacteria and amebas together might be responsible for some cases of suppurative periodontitis, but this conservative attitude was abandoned by Bass and Johns,[17] who stated that all cases of periodontal diseases were due to the action of amebas and that emetin was a specific cure. Talbot[18] and Smirnow,[8] among others, failed to confirm the specific role of either bacteria or amebas. In an excellent review, Glynn[19] pointed out that these protozoa probably act as scavengers and lack invasive power, since, they are never found in living periodontal tissues. This investigator also reviewed the published reports on the bacteriology of suppurative periodontitis in addition to his cultural findings in 20 cases of this disease and in 25 normal cases, and concluded that there seemed to be evidence that streptococci were the important aerobic pathogens. He indicated that fusiform bacilli and spirochetes might be pathogenic under some conditions.

In the opinion of Beckwith, Williams and Rose,[20] who recently reviewed the evidence for bacterial as well as local and general systemic factors in causation of suppurative periodontitis, infection play an important but secondary role. This viewpoint had been advanced earlier on more or less theoretical bases by Medalia,[21] Byrne,[22] Talbot[18] and others and probably represents the opinion of most investigators at the present time.

It is evident that for a consideration of the causation of this disease it would be necessary to have complete information on all the bacteriological as well as the nutritional and local factors which might be involved, since the complexity of the problem seems to increase with increasing knowledge in these fields. Almost all of the investigators of the bacterial flora of pus from periodontal pockets and, in fact, most of the workers concerned with the flora of normal mouths have used aerobic technics. As Wagner[23] and Gins[24] point out, there is a large and little understood group of anaerobic microorganisms whose distribution and pathogenicity in normal and abnormal conditions remain to be investigated by modern technics. This problem has been approached in the present study by 3 different methods, each to be presented in a separate section. First, in order to determine

10. Dental Cosmos **51:** 55, 1910.
11. Dental Cosmos **57:** 540, 1915
12. Dental Cosmos **55:** 388, 1913.
13. J. Am. Dent. A. **12:** 1452, 1925.
14. Am. J. Path. **3:** 169, 1927.
15. Rev. de stomatol. **31:** 215, 1929.
16. Dental Cosmos **57:** 1201. 1915.
17. J.A.M.A. **64:** 553, 1915.
18. J.A.M.A. **64:** 928, 1915.
19. Brit. Dent. J. **44:** 601, 1923.
20. M. J. & Rec. **129:** 333, 1929.
21. Dental Cosmos **55:** 24, 1913.
22. Dental Cosmos **57:** 1261, 1915.
23. Med. Klin. **30:** 553, 1934.
24. Zentralbl. f. Bakt. (Abt. 1) **132:** 129, 1934.

whether the anaerobic flora of the healthy gingival crevice differed in any important respect from that of the periodontal pocket in cases of suppurative periodontitis, cultures of these areas were made and compared. Secondly, the potential pathogenicity of the microorganisms present in periodontal pockets and normal mouths was investigated by animal inoculations. Finally, attempts were made to produce experimental suppurative periodontitis in monkeys by methods designed to create favorable conditions for multiplication of certain of the oral flora.

CULTURES

As one method of determining the importance of anaerobic bacteria in the development of suppurative periodontitis, a study of the cultivable flora of the normal and diseased gingival crevice was made. The distribution of the various groups of anaerobes recovered from these cultures was compared in order to find any qualitative or quantitative differences which might be correlated with the disease process.

Materials and methods.—The areas to be cultured were cleansed of superficial debris and pus which had been expressed during the examination. Specimens were taken with sterile root scalers so that calculus and granulation tissue were included as well as exudate. For comparison, material was removed in a similar manner from the normal gingival crevice of molar and premolar teeth in mouths free from gingival inflammation. Samples were streaked out on 10% sheep blood agar plates and incubated anaerobically according to the method described by Dack, Dragstedt and Heinz.[25] After 4 to 7 days at 37 C., the plates were examined microscopically and representative colonies were selected for staining. To determine which microorganisms were obligate anaerobes and which were only facultative, blood agar slants were inoculated from isolated colonies and incubated aerobically and anaerobically. A number of cultures were kept for further study and these were grown in deep tubes of dextrose brain medium.

25. J. Infect. Dis. **60:** 335, 1937.

Results.—The distribution of anaerobic bacteria found in normal and diseased gingival crevices is compared in table 1. Only those forms are listed which appeared in at least 20% of the cultures in either series. Only one other microorganism was found in more than

TABLE 1.—*The Most Common Anaerobic Microorganisms Obtained from Normal and Diseased Human Gingival Crevices*

Microorganism	Suppurative Periodontitis (46 Cases)		Normal (27 Cases)	
	Times Found	Per Cent	Times Found	Per Cent
Spirochetes*	46	100	11†	61
Fusiformis nucleatus	17	37	10	37
Other fusiform bacilli	10	22	3	11
M. gazogenes	22	48	19	70
Bact. melaninogenicum	13	28	1	3
Gram-positive cocci	11	24	7	26
Leptotrichia	10	22	7	26
Bacteroids	9	19	12	44

* Incidence determined from Giemsa stained smears and darkfield preparations; all others from cultures.
† Of 18 cases.

1 or 2 cases; this was a gram-negative filamentous rod which was recovered from 4 diseased and 3 normal mouths, but this was considered to be of doubtful importance because of its low incidence.

It may be seen that the flora of pus from periodontal pockets differed quantitatively from that of the normal gingivae but no qualitative difference was found. Bacterium melaninogenicum, although found in only one culture of the normal gingival crevice in this series, probably is present in small numbers in most mouths. Burdon[26] was able to isolate this microorganism constantly from normal gingivas and found that poor oral hygiene created favorable conditions for its multiplication. Additional evidence has been obtained on this point from other studies which are described elsewhere in this paper.

Although found in some normal mouths, fusiform bacilli,[27] like Bact.

26. J. Infect. Dis. **42:** 161, 1928.

27. For a brief review of the literature on classification of oral fusiform bacilli, see Hine, M. K., and Berry, G. P.: J. Bact. **34:** 517, 1937.

melaninogenicum, find a more favorable environment in the periodontal pocket, as evidenced by the number of cases from which they were cultured. Examination of Giemsa stained smears of the original material before culture revealed the presence of fusiform bacilli more often and in greater numbers than was evident from the cultural results. Although this observation showed that complete reliance cannot be placed on the culture method employed for the isolation of fusiform bacilli, it did confirm the fact, indicated by the cultural findings, that the increased incidence of these forms was relative. The examination of dark-field preparations, as well as the Giemsa stained smears, showed that the same apparently was true of spirochetes. These findings confirmed the results of other investigators of normal mouth flora, including Mühlens and Hartmann,[28] Ozaki[29] and Brooke,[30] and the observation of increased incidence of these forms in periodontal lesions as compared with normal mouths was in accord with the results of Howitt and Fleming[31] and Smith.[32]

The incidence of other anaerobic microorganisms, Micrococcus gazogenes, gram-positive cocci, leptotrichia and bacteroids (gram-negative rods)[33,34] appeared to be somewhat less in diseased than in normal mouths, although the differences were not striking.

Filamentous, nonbranching rods resembling Leptotrichia, according to the criteria given by Bergey,[35] were found in a large percentage of smears from normal mouths. This observation was made also by Brailovsky-Lounkevitch[36] and Bibby.[37] A similar high incidence of these forms has been obtained in the two series reported here, but the number of successful cultures has been small. No attempt has been made to relate any of the types recovered in this study to those reported by others.[38]

The occurrence of anaerobic, nonhemolytic gram-positive cocci, especially streptococci of the type described by Veillon[39] in normal and diseased body cavities, has been reported by various investigators, but no systematic study of these forms as they occur in the mouth has appeared. Lewkowicz[40] was the first to isolate a streptococcus which has been called Streptococcus micros from mouths of suckling infants. Different strains similar to Prevot's[41] Streptococcus intermedius were isolated by Taylor[42] from cases of "dental sepsis." Bergey[35] listed several other species which were said to inhabit the normal oral as well as the intestinal and genital mucous membranes. In pure culture these microorganisms seem to have little invasive power, according to Taylor[42] and McDonald, Henthorne and Thompson,[43] but in certain conditions in association with other bacteria they are

28. Ztschr. f. Hyg. u. Infektionskr. **55:** 81, 1906–1907.

29. Zentralbl. f. Bakt. (Abt. 1) **62:** 76, 1912.

30. J. Dent. Research **17:** 57, 1938.

31. J. Dent. Research **10:** 33, 1930.

32. Oral Spirochetes and Related Organisms in Fusospirochetal Disease, Baltimore, Williams and Wilkins, 1932.

33. Henthorne, J. G., Thompson, L., and Beaver, D. C.; J. Bact. **31:** 255, 1936.

34. Varney (Arch. Surg. **19:** 1602, 1929) isolated these forms from lung abscesses.

35. Bergey's Manual of Determinative Bacteriology. ed. 5, Baltimore, Williams and Wilkins, 1939, p. 281.

36. Ann. Inst. Pasteur **29:** 379, 1915.

37. A Study of the Filamentous Bacteria of the Mouth, Thesis, University of Rochester, 1935.

38. Bibby, B. G., and Berry, G. P.: J. Bact. **38:** 263, 1939.

39. Compt. rend. Soc. de Biol. **45:** 807, 1893.

40. Arch de med. expér. et d'anat. path. **13:** 633, 1901.

41. Ann. Inst. Pasteur **39:** 417, 1925.

42. A System of Bacteriology in Relation to Medicine. London, His Majesty's Stationery Office, 1929, vol. 2, p. 136.

43. Arch. Path. **23:** 230, 1937.

able to produce putrid, gangrenous lesions in various tissues of the body. It would appear that whatever those conditions might be, they do not obtain to any marked degree in periodontal pockets, since these organisms were found in only about one-fourth of the cases cultured. However, the observed incidence may have been somewhat low since, as pointed out by Taylor, growth of these forms in primary culture is slow and they might have been overlooked because of overgrowth by aerobic and other anaerobic bacteria present in the inoculum.

One of the few anaerobic species found in this study which is thought to inhabit the oral cavity exclusively is M. gazogenes. This small gram-negative coccus was first isolated by Lewkowicz[40] from the mouth of an infant. More extensive studies of the morphological and biochemical characteristics of this organism have been made by Hall and Howitt[44] on 24 strains from human saliva, and by Prevot[45] on 15 strains from various sources, including the mouth. Bergey[35] listed 3 varieties of the type species, gingivalis, minutissma and syzygios. The last named was found by Herzberg[46] in 30% of normal mouths and also in 100% of salivas from scarlet fever patients. A study of the biochemical properties of 22 strains of M. gazogenes isolated from both normal and diseased mouths in this investigation indicated that 19 of these could be classed as var. syzygios on the basis of reduction of nitrate and production of small amounts of H_2S. The position of the other strains has not been determined but there was no correlation, within this group, of metabolic activity with the condition of the mouth from which the strains were isolated. Two strains of this coccus, obtained through the kindness of Dr. Ivan C. Hall for comparison with our cultures, were found to be similar in morphologic and cultural characteristics to the majority of our strains and to fall into the same variety.

Study of the pathogenicity of this microorganisms for several laboratory animals gave results similar to those reported by Hall and Howitt.[44] Mice inoculated subcutaneously, intraperitoneally or intravenously with several strains showed no evidence of progressive infection, but animals killed within 3 or 4 days following intravenous injection had minute abscesses in the liver and, in several instances, also in the lungs. However, no microorganisms were isolated from cultures of the abscesses. Histopathological examination of the tissues showed areas of acute focal inflammation but the staining technic was inadequate for demonstration of gram-negative bacteria which may have been present in the lesions. Mice killed at intervals longer than 4 days showed no pathological changes. Further evidence of lack of independent pathogenic activity was obtained by inoculation of pure cultures beneath the gingival mucosa of 2 monkeys. Only a transient inflammation was produced and in 1 animal a small area of necrosis was found which did not break down but healed in about 4 days. This monkey was deficient in the M factor of Langston et al.[47]

Differentiation of these strains according to the immunological groups described by Hall and Howitt[44] was attempted with serums prepared against 4 strains, 1 each from a normal and a diseased gingival crevice and the 2 strains obtained from Dr. Hall. Considerable difficulty was experienced in pre-

44. Proc. Soc. Exper. Biol. & Med. **22:** 542, 1913.

45. Ann. Sc. nat., sér. bot, **15:** 127, 1933.

46. Zentralbl. f. Bakt. (Abt. 1) **111:** 373, 1929.

47. J. Exper. Med. **68:** 923, 1938.

paring stable suspensions of some strains but satisfactory tests were completed in most instances. The agglutination reactions obtained were, however, mainly strain specific and no evidence was found of any serological types within the group of strains studied.

In addition to being the commonest organism found in the series of cultures from normal mouths and suppurating periodontal pockets, M. gazogenes often was the predominating anaerobic form cultured from an individual sample, although spirochetes were the most numerous organisms found in smear and darkfield preparations of pus from periodontal pockets. On the other hand, leptotrichia-like filamentous rods usually predominated in smears from normal gingival crevices and spirochetes and, when present, were few in number. When M. gazogenes was not found, anaerobic, gram-positive cocci predominated in the culture, often closely followed by fusiform bacilli. The other anaerobes, especially Bact. melaninogenicum, were found in very small numbers.

ANIMAL INOCULATIONS

Since no one microorganism seemed to be specifically associated with suppurative periodontitis, although there was a relative increase in numbers of several members of the flora of the normal gingival crevice, it seemed possible that an associative or symbiotic relationship existing between these forms might be important in the pathogenesis of the disease. Examples of such associations of mouth organisms are not unknown, but usually they have been implicated in such serious conditions as Vincent's angina,[48] lung abscesses,[49] or gangrenous processes of the soft tissues of the face or other portions of the body.[50]

In general, the groups of fusiform bacilli and spirochetes have been the most consistently observed components of the flora of these lesions, but various aerobic and anaerobic bacteria always accompany them. Several species of cocci often have been recovered (Kritchevsky and Seguin,[51] Davis and Pilot[52]) together with filamentous rods (Silberschmidt[50]) and vibrios (Veszpremi,[49] Rosebury and Foley[48]) or bacteroids (Weiss and Shevky[53]). These associations were demonstrated by inoculation of pus or exudate from the pathological process into animals and examination of the lesions produced. The results of such injections as reported by different investigators varied from localized abscess formation which was followed by regression, to progressive, gangrenous infections which could be passed in series, with an increase in virulence (Veszpremi[54]) or a decrease (Silberschmidt[50]) on continued passage. Using material from a pulmonary abscess, Kline[55] was able to pass the infection through guinea pigs in series, but after 12 transfers the spirochetes disappeared, although the infection still could be transmitted. Smith[32] concluded from the results of inoculation of guinea pigs with pure cultures, individually and in combination, of Treponema microdentium, a fusiform, an anaerobic streptococcus and Vibrio viridans, that these 4 organisms together are responsible for gangrenous mouth lesions, including suppurative periodontitis. In some cases

48. Rosebury, T., and Foley, G.: J. Am. Dent. A. **26:** 1798, 1939.

49. Veszpremi. D.: Zentralbl. f. Bakt. (Abt. 1) **44:** 332, 408, 515 and 648, 1907; **45:** 15, 1907–1908.

50. Silberschmidt, W.: Zentralbl. f. Bakt. (Abt. 1) **30:** 159, 1901.

51. Dental Cosmos **63:** 888, 1921.

52. J.A.M.A. **79:** 944, 1922.

53. Arch. Path. **22:** 770, 1936.

54. Zentralbl. f. Bakt. (Abt. 1) **38:** 136, 1905.

55. J. Infect. Dis. **32:** 481 1923.

it was necessary to deplete the animals in vitamin C before the inoculation would take effect; this was especially true when microorganisms recovered from cases of noma were used.

In general, however, periodontal pus when inoculated into normal animals has been found to produce only localized abscesses which heal within a few days (Kritchevsky and Seguin,[51] Weiss and Shevky[53]) but no reports of attempts to pass such material in series in animals have been found.

Materials and methods.—Pus, obtained as already described, was collected in about 0.5 cc. of veal infusion broth or ascitic fluid. The masses of calculus and exudate were broken up with a glass rod and the suspension was injected subcutaneously and intratesticularly into normal rabbits, intraperitoneally, intravenously and subcutaneously into mice, and into the gingival mucosa of a monkey. With the exception of the intratesticular injection of the rabbit, the inoculations were repeated several times, using material from 4 different periodontitis cases. Cultures, darkfield examinations and smears for staining were made from each sample. The flora of these cases did not differ from that of the other diseased mouths examined and the cultural results were included with the others in table 1. Portions of some of the samples were passed through sintered glass filters and inoculated intratesticularly into rabbits and intracerebrally into mice.

The animals used in the experiments described above were healthy and were maintained on the laboratory diet, with a single exception noted elsewhere. In addition, guina pigs, fed for 1 week to 10 days on a diet identical with the "vitamin M" deficient diet no. 600 of Langston, Darby, Shukers and Day[47] (with the exception that ascorbic acid was not added), were inoculated subcutaneously with pus from periodontal pockets. On this regimen, the animals exhibited the usual features of vitamin C deficiency, such as weight loss, roughening of the coat and reddening of the oral mucous membranes, within 10 days and usually were dead within 2 weeks as the result of the deficiency if not killed sooner by infection. In order to control the dietary experiment in respect to the M deficiency, 4 guinea pigs was placed on the diet with adequate supplement of vitamin C. Three of these animals died in 4 to 6 weeks with diarrhea and the other remained healthy. This guinea pig reacted in the same manner as a normal animal to inoculation with pus. For this reason and because the effects of the C deficiency were so acute as compared to those of M deficiency, it seemed likely that the lack of vitamin M had little effect on the progress of the infection.

The size of the inoculum varied, depending on the amount of material obtained, the route of inoculation and the animal used. For subcutaneous injections, from 0.1 cc. to 0.3 cc. was used, the larger amounts for rabbits and the smaller for mice. Intraperitoneal doses were from 0.15 to 0.4 cc., and 0.5 cc. was injected intravenously. Mice received 0.03 cc. of filtrate intracerebrally, and 0.4 cc. of the same material was inoculated into the testicles of rabbits.

Results.—Small, well localized abscesses developed at the site of 1 intratesticular and 4 subcutaneous injections of normal rabbits, but no progressive infections resulted. Rapid healing followed breakdown and discharge of pus from the lesions and usually the animals had completely recovered within a week or 10 days after inoculation. Attempts to infect 1 normal rabbit each with pus from the subcutaneous or testicular abscesses were unsuccessful.

Similar results were obtained by subcutaneous inoculations into 3 mice, and, furthermore, no localized or generalized infections were produced in 2 experiments with intravenous injection of material from suppurating pockets into these animals. No evidence of peritonitis was found after intraperitoneal inoculation of periodontal exudate into 3 mice, but the animals usually died in about 48 hours, possibly from a toxemia similar to that observed by Beckwith, Williams and Rose[20] after injection of heated suspensions of exudate from periodontal pockets.

The monkeys which received injections of periodontal exudate into the gingival mucous membrane developed small areas of necrosis which broke down and discharged some purulent exudate, but healing was complete in about a week. One of these animals was deficient

in the M factor of Langston et al.[47] and the other was normal.

No evidence of the presence of a virus in filtrates of suspensions of pus from periodontal pockets was obtained by 3 serial intracerebral passages in mice. Seven days intervened between passages and one-half of the brain was used in a 20% suspension in broth for transfer. Inclusion bodies were not found in

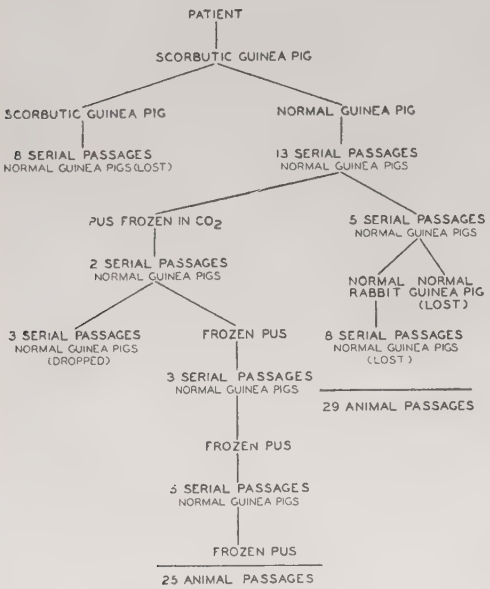

Fig. 1.—Animal passage of pus from a case of suppurative periodontitis.

Giemsa stained impression smears of rabbit testicles which had been injected with the filtrate.

Although normal animals seemed relatively resistant to subcutaneous inoculation with periodontal exudate, scorbutic guinea pigs, similarly injected, died with a progressive infection including invasion of the blood stream after as short a period as 2 days, or less often after 10 days to 2 weeks. In the latter case death probably was due to dietary deficiency. By inoculation of normal guinea pigs with exudate from the lesions of deficient animals an infection was produced which was passed in series (fig. 1) with progressive increase in virulence and decrease in complexity of the flora until a stable bacterial association was evolved. It appeared that no difficulty would be encountered in maintaining the infection indefinitely if desired. Rapid freezing and storage at −70 C. preserved infective material for as long as 24 days with only slight loss of infectibility and no qualitative changes in the flora.

In the early passages, pus inoculums were relatively large, usually from 0.2 cc. to 0.5 cc., and death was often delayed for a week or 10 days after inoculation. Infections which terminated fatally in about 48 hours were commonly induced in the later passages by injection of as little as 0.03 cc. of material. Mice inoculated subcutaneously with pus from early passage guinea pigs frequently died of peritonitis resulting from direct extension of the abscess through the body wall, but with pus from a late passage, death of these animals was more often due to the same type of spreading infection as was observed in the guinea pigs. A normal monkey inoculated into the gingivae with pus from a late passage developed a marked cellulitis of the lip which drained and healed in about a week. Another monkey, which was deficient in M factor and which exhibited a gingivitis referable to the deficiency, when similarly inoculated developed a suppurative lesion superimposed upon the gingivitis and also suffered from a cellulitis of the upper lip. These effects persisted for about 2 weeks, at which time the animal died without any evidence of spread of the infection from the draining upper lip, so that death was presumed to be due to the deficiency. Pus obtained from this monkey on the 13th day of the infection failed to induce any visible infection in a normal guinea pig.

Subcutaneous inoculation of guinea pigs with pus from a late passage produced an acute infection with extreme prostration and death within 48 hours. The phlegmon spread rapidly over the ventral surface of the animal and usually involved most of the subcutaneous tissues of the chest and abdomen at death. Externally, a hemorrhagic discoloration of the skin and edematous swelling marked the extent of the lesion. When the skin was reflected, either a slightly blood tinged, watery fluid or a purulent exudate was found. The nature and extent of this exudate seemed to depend on the duration of the infection. There was marked injection of the subcutaneous vessels. A grayish-green, necrotic area at the point of inoculation usually was present. With gentle traction the muscles of the abdomen and chest could be pulled away leaving ragged shreds of necrotic tissue. The visceral changes were slight and, at most, consisted of swelling and hemorrhage into the adrenals and a slight pneumonic consolidation. The exact cause of death was not apparent, but the changes in the adrenals suggested a toxemia. Blood cultures were positive in the terminal stages of the disease, but whole blood did not transmit the infection. The results of the blood cultures are discussed elsewhere.

The flora of the pus as determined at each transfer by anaerobic blood agar cultures, Giemsa stained smears and darkfield preparations, was observed to change during the first 7 passages from the complex group of bacteria present in the original material from the patient to a stable, relatively simple association which remained unaltered during subsequent transfers (table 2). The first 2 passages in scorbutic animals eliminated the least adaptable members of the flora, but did not seem to affect the spirochetes, of which several morphological types were present. After 7 serial passages only 1 spirochete remained. This resembled T. microdentium. Since differentiation of the types of spirochetes was made only on the basis of morphology in darkfield and smear preparations and comparison with descriptions and illustrations of Noguchi[56] and Smith,[32] the designation of species listed in the table should be regarded as only tentative. All of the bacterial species surviving animal passage have been found in cultures of normal as well as diseased gingival crevices, and the relative incidence of some of these has been described (page 133, et seq.). Only obligate anaerobes were included in the description of the cultural findings but many species of facultative anaerobes also were found, including vibrios and various types of cocci. No separate determination of the incidence of these forms was made.

TABLE 2.—*Simplification of the Flora of Periodontitis Pus by Animal Passage*

Microorganisms	Patient	Passage in Animals	
		Scorbutic*	Normal†
Vibrio sputigenus	+	+	+
Staph. albus	+	+	+
Gram-positive diplococcus	+	+	+
Streptococcus (gamma)	+	+	+
Bact. melaninogenicum	+	+	+
Gram-positive filamentous rod	+	+	+
Fusiform bacilli (unidentified)	+	+	+
T. microdentium (?)	+	+	+
T. macrodentium (?)	+	+	0
T. vincenti (?)	+	+	0
Leptospira (?)	+	+	0
Fusiformis nucleatus	+	0	0
Diphtheroid	+	0	0
Streptococcus (alpha)	+	0	0
Neisseria sp.	+	0	0
Micrococcus sp.	+	0	0

* First and second serial passages.
† After seventh serial passage.

In order to assess the importance of each organism present in the association, it would be necessary to have pure cultures of all of the forms and to test their ability in pure and mixed culture to produce the characteristic lesion. It

56. J. Exper. Med. **15**: 81, 1912.

was not possible to do this since some of the bacteria, that is, the spirochete, fusiform bacillus, filamentous rod and Bact. melaninogenicum, were not separately isolated. However, a little information has been obtained on this point and is presented in table 3.

Cultures of the passage pus were made in Smith-Noguchi[32] medium, which consists of semisolid ascitic fluid agar

TABLE 3.—*Animal Inoculations with Cultures from Passage Pus*

Organisms Persisting in Animal Passage	Culture Mixtures Inoculated		
	A*	B*	C*
Vibrio sputigenus	+	+	+
Staph. albus	+	+	+
Gram-positive diplococcus	+	+	+
Streptococcus (gamma)	+	+	+
Gram-positive filamentous rod	+	+	0
Bact. melaninogenicum	+	0	0
Fusiform bacilli (unidentified)	+	0	0
T. microdentium (?)	+	0	0
Results of inoculations	Rapidly spreading lesion fatal in 48 hours	Slowly spreading lesion fatal in 11 days	No lesion

* A = total growth on a blood agar plate, B = growth in Noguchi medium, and C = mixed pure cultures.

with a piece of sterile rabbit kidney added. Incubation was continued for 21 days, as recommended by Smith,[32] and suspensions of the growth were injected subcutaneously into normal guinea pigs. A slowly progressive, somewhat purulent, infection was produced in 4 different experiments and terminated fatally after a period of 3 to 11 days. Fusiform bacilli and spirochetes could not be demonstrated in the culture nor in the guinea pig lesions and they probably were not present, since they did not appear even after further passage of the infection. The black pigment characteristic of Bact. melaninogenicum could be seen in the cultures, but this organism did not grow in subcultures nor was it found in the lesions of the animals. In order to rule out the possibility that some toxic material in the culture medium was responsible for part or all of this progressive lesion, passage was made in series through 3 normal animals with the reproduction each time of the same type of infection. This result seemed to indicate that, while the presence of the fusiform bacillus, spirochete and Bact. melaninogenicum might be necessary for the characteristic, acute, fatal infection, the other members of the flora also had some invasive power.

Mixed pure cultures of the vibrio and cocci isolated from anaerobic blood agar cultures of passage pus produced no lesions in the guinea pig. These organisms were recovered from blood cultures of guinea pigs in the terminal stages of the disease, but, as previously indicated, whole blood was not capable of inducing an infection in another animal.

The fact that vibrios and cocci when introduced into an animal together with a filamentous rod (Smith-Noguchi culture) were able to survive and multiply, but in its absence were destroyed by the normal defenses of the body, would assign to it an important role in the pathogenesis of the infection. However, the possibility should not be overlooked that after they became established vibrios and cocci might have played an important part in the spread of the lesion. These organisms were always present in much greater numbers in the exudate than any other members of the flora.

In 3 different experiments suspensions of the total growth of passage pus on anaerobic blood agar plate cultures produced in normal guinea pigs lesions indistinguishable from those produced by transfer material. The characteristic flora was demonstrated on these plates, as well as in material from the guinea pigs inoculated with the suspensions. The flora included the spirochete, which evidently was able to survive and multiply in the heavily seeded area of the plate. This infection was readily passed in series in normal animals.

The experimental transfer of periodontal pus through animals has been repeated, using material from 2 additional patients, with results similar to those described above. The changes noted in the bacterial flora of material from these 2 periodontitis cases were the same as those described previously and the final flora differed only in the presence of alpha hemolytic streptococci and, in 1 case, beta hemolytic streptococci also were found.

In order to determine whether the flora of the normal gingival crevice produced the same results if handled in a similar manner, passage experiments were made with material from the mouths of 2 persons having normal gingivae. The infections were much slower to develop than was the case with the periodontal exudate, and spontaneous healing of the lesions was the general rule. After a relatively large series of transfers (5–7), the material induced lesions similar to those described in the periodontitis series. The changes in bacterial flora associated with passage were the same as those described previously; alpha and beta hemolytic streptococci again were present. In both of these experiments Bact. melaninogenicum was cultivated from the lesions of the passage animals althought it was not found in the original culture from the gingival crevice of either of the 2 persons. As suggested previously, this microorganism probably was present on the normal gingivae in too small numbers for detection by the cultural method used.

The observations of Smith[32] on the pathogenicity of a combination of pure cultures of a vibrio, a spirochete, a fusiform bacillus and an anaerobic streptococcus isolated from the mouth represent an approach to the same problem of the invasive power of the mouth flora from the reverse direction. In the experiments described above, starting with periodontal exudate instead of pure cultures, the same 4 groups of microorganisms were found in an association capable of infecting guinea pigs, together with others of more or less importance as indicated. It would appear from our results that the filamentous rod which Smith did not study was able to substitute, at least in part, for the fusiform bacillus and spirochete in the microbic combination. It also seems possible that a number of species of streptococci and micrococci may take the place of the anaerobic streptococcus in the association.

EXPERIMENTAL SUPPURATIVE PERIODONTITIS

In spite of the fact that suppurative periodontitis is said to occur in various species besides man (Smith,[32] Leary,[10] Williams[57]), experimental production of the disease in laboratory animals has been reported in only 3 instances, none of which has been confirmed. Mendel[58] inoculated a small series of rabbits with mixtures of Staph. parvulus, a diphtheroid, a gram-negative sporulating rod and a vibrio isolated from persons with periodontitis. Subcutaneous injection of this mixture produced no effect but when introduced beneath the gingivae, a chronic suppurative process resembling periodontitis was induced in 4 of 8 animals. The other rabbits showed more extensive involvement. By subgingival injection of pure cultures of a nonhemolytic streptococcus isolated from cases of this disease, Smirnow[8] was able to induce suppuration of the gingivae of cats. According to Smith,[59] oral inoculation of periodontal exudate into scorbutic guinea pigs was followed by development of the clinical picture of suppurative periodontitis.

57. Am. J. Med. Sc. **141:** 666, 1911
58. Compt. rend. Soc. de biol. **80:** 962, 1917.
59. J. Am. Dent. A. **23:** 1340, 1936.

It would seem, however, that if spontaneous periodontitis does occur, it does not necessarily represent the introduction from without of some bacterial species which are able to produce the disease. At least as logical an assumption might be that a process occurs in animals similar to that which is thought by some to take place in man. As suggested by Goadby,[1] lowering of the resistance of the periodontal tissues under certain conditions may create a favorable environment for the multiplication of some of the flora of the normal gingival crevice. These organisms might contribute to the gingival inflammation which is a part of the picture of suppurative periodontitis.

The importance of vitamin deficiencies as predisposing factors in periodontal disease has been stressed in the experimental work of Smith[32] and a number of other investigators. A gingivitis, in addition to anemia, leukopenia and diarrhea was noted by Langston, Darby, Shukers and Day[47] as characterizing deficiency in a factor designated by them as "vitamin M." The effects of the lack of this substance in the diet were cured by yeast or liver. These observations have been confirmed by Topping and Fraser.[60] The latter also used diets deficient in other vitamins and found that gingival inflammation was produced by a number of other avitaminoses. From the mouth lesions in monkeys they obtained smears which showed large numbers of fusiform bacilli and spirochetes.

In order to study the relation of the oral flora of monkeys to gingival inflammation and to explore the possibility of inducing a suppurative periodontitis in these animals, a group of monkeys was placed on the diet described by Langston, et al.

60. Pub. Health Rep. **54**: 416, 1939.

Materials and methods.—A total of 36 monkeys was studied of which 6 were normal controls, 3 were fed the ordinary laboratory diet and 3 were on the experimental diet supplemented with cod liver oil, ascorbic acid and yeast. Preliminary blood counts, weights and cultures of the gingival crevice were taken and with the exception of the blood counts were repeated biweekly throughout the course of the experiment. Blood counts were made once a month unless there was evidence of severe illness. Fifteen of these animals were used to study the relation of the M factor to infection with Bact. dysenteriae (Flexner).[61]

Results.—The findings in these monkeys were, in general, less striking than those reported by Langston, et al. but they followed the same pattern. Anemia was not marked but the white count dropped to 5,000 or below in several instances. The usual normal white count was found to be about 10,000 to 15,000, but occasionally was observed to be as high as 28,000 per cm. The diarrhea which proved to be referable to a Flexner dysentery infection complicated the picture in 10 of a group of 15 of the monkeys in which this aspect of the problem was studied.[62] Some of these animals developed high white cell counts after an initial drop, due probably to the superimposed infection. Severe weight losses occurred in every animal.

Of the 30 monkeys on the experimental diet, 11 developed gingivitis. In the majority of cases the involvement was slight, consisting of some localized inflammation of the gingival margins. One animal which developed a nutritional edema showed slight, superficial ulceration of the gingivae but this was the most extensive lesion noted in the series.

A few monkeys in this group were placed on the basic diet without either

61. Janota, M.: Experimental Bacillary Dysentery and Spontaneous Development in Monkeys on Vitamin M Deficient Diets, Thesis, University of Chicago, 1940.

62. Janota, M., and Dack, G. M.: J. Infect. Dis. **65**: 219, 1939.

supplemental cod liver oil or ascorbic acid or both. No increased incidence or severity of the gingivitis was noted in these animals which, in addition to being deficient in the M factor, were lacking vitamins A and D, C, or all 3. It was not possible to test the effects of withdrawal of the B complex in these animals since the basic diet could not be modified to this extent. The daily addition of 10 gm. of yeast to the supplemented diet completely abolished the gingivitis within a day or two and the same effect was noted in a deficient monkey which was fed 2 slices of white bread a day.

As previously mentioned, the inoculation of periodontal exudate or pure cultures of M. gazogenes into the gingivae of these deficient monkeys produced only transient inflammatory reactions. Inoculation of a deficient animal with pus from a passage guinea pig in the series started from the first periodontitis case induced a marked suppuration which lasted longer and was somewhat more progressive than the lesion in the normal animal.

In general, the results of cultures of the normal and inflamed gingival crevices of these monkeys (table 4) were very similar to those of the human mouth. Anaerobic gram-positive cocci and bacteroids were omitted from this table since no differentiation was made by subcultures between obligate and facultative anaerobes.

The same general variations were seen in the incidence of fusiform bacilli, leptotrichia and M. gazogenes which had been noted in the cultures from normal and diseased human gingival crevices but the differences were much smaller. This seems to be due to a higher incidence of all of the cultivable forms in normal monkey than in normal human mouths. Especially notable was the high incidence of Bact. melaninogenicum but, unlike the findings in human mouths, no increase in the numbers of this microorganism was noted in the inflamed gingival crevice. Leptotrichia were isolated more than twice as frequently from monkey than from human mouths.

No lesions simulating suppurative periodontitis were observed in any of these animals, and in only one case was there a suggestion of a periodontal pocket with, however, no suppuration.

TABLE 4.—*The Most Common Anaerobic Microorganisms Cultivated from Normal and Inflamed Gingival Crevices of Monkeys*

Microorganism*	Condition			
	Gingivitis (11 Cases)		Normal (37 Cases)	
	Times Found	Per Cent	Times Found	Per Cent
Fusiformis nucleatus	4	36	10	27
Other fusiforms	2	18	9	24
M. gazogenes	3	27	13	35
Bact. melaninogenicum	5	45	21	57
Leptotrichia	7	64	23	61

* Since no differentiation was made between facultative and obligate anerobes by means of subcultures, gram-positive cocci and bacteroids were omitted from this table.

Attempts were made to induce pocket formation in a monkey by separation of the gingivae from several teeth by means of a blunt scaler, but the lesions healed rapidly. It seemed that a more chronic type of irritation was necessary to insure permanence of the artificial pocket, and since it is a clinical as well as an experimental observation that alteration of occlusal stress will often induce the formation of a pocket, this method was adopted.

Three monkeys were used for this study. Occlusal relations of one or more teeth were altered by means of improperly contoured fillings and crowns and also by altering the occlusal surfaces and proximal contact points by grinding. In some cases the restorations were prepared with increased vertical height in order to produce traumatic occlusion—a clinical condition believed by many to be an important factor in periodontal

disease in man. Dr. J. R. Blayney, Dr. H. P. Steinmeyer, and Dr. T. G. DeWitt assisted in planning and executing the operative procedures and x-ray examinations of these animals One of these animals died after about 6 weeks at which time a shallow pocket was found with an explorer, but no x-ray evidence of alveolar bone resorption was present. After a similar period, the 2 remaining monkeys were put on the M factor deficient diet. Both died after about a month of the dietary regimen and shallow pockets were present between their gingivae and teeth. There was slight gingival inflammation about the operated tooth of one animal but neither showed any evidence of suppuration.

The methods used in this study apparently were not suitable for the experimental production of suppurative periodontitis although 2 antecedent conditions, pocket formation and gingivitis were established, in 1 case in the same mouth. Probably there are other factors necessary for the production of the full clinical picture of this disease, and at least some of these might be suitable for experimental study. It seems unlikely, however, that these factors include alteration of the anaerobic bacterial flora of the monkey gingival crevice since this appears to be similar to that found in the human disease.

DISCUSSION

In this investigation the flora of suppurating periodontal pockets has been compared in respect to its distribution and potential pathogenic activity with the anaerobic bacterial population of the normal human gingival crevice. A relative increase was found in the incidence in the periodontal exudate of certain bacterial species, namely, fusiform bacilli, spirochetes and Bact. melaninogenicum, all of which were found in small numbers in some normal mouths. These same microorganisms in association with several species of facultatively anaerobic cocci, a vibrio and a filamentous rod were demonstrated in lesions in guinea pigs produced by serial transfer of pus from periodontal pockets and material from normal gingival crevices. The results of these cultural and pathogenicity studies on the anaerobes present in the 2 environments suggest that, while the bacterial flora is qualitatively similar, the quantitative differences observed result from adaptation of the microorganisms of the normal gingival crevice to conditions in the periodontal pocket. The increased opportunity for multiplication afforded by the changes in surroundings to this association of bacterial species also may allow these organisms to acquire somewhat increased invasive powers. This is evidenced by their ability to produce spreading, fatal infections in guinea pigs with a minimum of experimental manipulation as compared to the same forms found in normal mouths. The invasion of normally resistant animals, which took place relatively more slowly when passages were made only in normal guinea pigs, was facilitated by preliminary passage through scorbutic animals. Hence, the initial virulence of the bacterial association may be said to have been further increased by growth in tissues of animals whose resistance had been lowered by vitamin C deficiency.

With these observations in mind, the relation of these findings to the problem of pathogenesis of suppurative periodontitis may be considered. From the fact that the flora of periodontal pockets is the same as that of the normal gingival crevice, although changed somewhat in relative proportions, it would seem likely that bacterial invasion could not be the primary factor in causation of this disease. Rather, other local or systemic

conditions which cause breakdown of periodontal tissues with formation of "pockets" between the teeth and the gingivae must be responsible for the early changes in periodontitis. On the other hand, these factors, whatever they may be, incidentally create favorable conditions for the multiplication of certain of the flora of the normal gingival crevice, thus producing the changes in incidence of these forms noted in the cultural studies. As indicated by the progressive enhancement of virulence in the experiments in animals, it might be possible for these bacteria, once they have invaded the diseased and therefore less resistant tissues, to acquire sufficient virulence to spread into the adjacent healthy tissues and produce a chronic suppurative infection. The role of anaerobic bacteria in the pathogenesis of suppurative periodontitis then may be conceived to be that of secondary invaders which, however, may be responsible for the suppuration which is so characteristic of this disease. This view is held by many periodontologists on the basis of clinical observations.

The role of vitamin C deficiency in promoting the invasive power of the bacterial association present in periodontal exudate seems to be in its ability to lower the resistance of the animals to infection. A similar lowered resistance was noted by McCullough[63] with Bact. necrophorum in these animals. Smith[59] claims suppurative periodontitis in scorbutic guinea pigs by oral inoculation of periodontal exudate. A suggestion that avitaminosis C may be an important factor in producing the pathological changes characteristic of periodontitis in man is found in the studies made by Boyle, Bessey and Wolbach.[64] These workers described the occurrence of alveolar bone atrophy in scorbutic guinea pigs with histopathological changes similar to those seen in the human disease. They reported no bacteriological findings on these animals. However, since extensive or conclusive studies of the blood ascorbic acid levels in periodontitis patients have not been reported, it is not known whether vitamin C deficiency plays a role in persons suffering with this condition. On the other hand, it has been stated[32] that lack of vitamin D and disturbance in calcium metabolism may contribute to the breakdown of periodontal tissue. Similarly, the importance of the B_2 complex in this initial phase of the disease is suggested by the pathological studies of Tomlinson[65] on the deficient monkeys of Topping and Fraser.[60] It is possible, of course, that a combination of these or other avitaminoses may be involved. After the pocket is formed, whether through the agency of one or several avitaminoses or other factors, it might be possible for invasion to take place by bacteria, thus converting the condition into a chronic suppurative infection. If the interpretation based upon the animal studies may be applied to this stage of the human disease, vitamin C or some other deficiency superimposed upon the conditions responsible for the initial lesion may make possible a more widespread and rapid establishment of the chronic suppurative state, which is characteristic of this disease.

63. J. Infect. Dis. **63:** 34, 1938.

64. Proc. Soc. Exper. Biol. & Med. **36:** 733, 1937.

SUMMARY AND CONCLUSIONS

Study of the bacterial flora of diseased and normal gingival crevices by means of anaerobic cultures, Giemsa stained smears and darkfield examinations has shown increased incidence of fusiform bacilli, spirochetes and Bact. melanino-

65. Pub. Health Rep. **54:** 431, 1939.

genicum in pus from periodontal pockets, as compared with material from normal mouths.

Serial transfers of pus from periodontal pockets demonstrated the presence of an association of microorganisms in this material which was able to produce progressive, phlegmonous lesions in guinea pigs. Included in this combination, in addition to the 3 anaerobic forms mentioned above, were several species of facultatively anaerobic cocci, Vibrio sputigenes, and a filamentous rod. These facultative anaerobes were found commonly in normal mouths as well as in the pus from periodontal lesions.

Material from normal gingival crevices also was passed in guinea pigs but the resulting infection was much slower to develop and tended to remain localized. Similar changes also were found in the flora of this passage pus, and the association which finally became established did not differ essentially from that which evolved in the periodontitis series.

Attempts to produce experimental periodontitis in monkeys by means of vitamin deficiencies known to induce gingivitis, together with artificial pocket formation, were unsuccessful. The flora of the gingivae of such monkeys was qualitatively similar to that of human periodontal pockets but a higher incidence of Bact. melaninogenicum and leptotrichia was found in monkey than in human mouths.

From the results of cultural studies and animal inoculation experiments it is believed that the role of bacteria in suppurative periodontitis is that of secondary invaders which may be responsible for the suppurative phase of the disease.